ON A POSSIBLE ROLE OF IMP IN THE REGULATION OF PHOSPHORYLASE ACTIVITY IN SKELETAL MUSCLE

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1. Introduction

In skeletal muscle, the transition from rest to exercise is associated with an accumulation of IMP. Concentrations of 2–4 mM IMP can be reached in perfused hind leg and of 1–2 mM IMP in hind leg in situ [1,2]. During exercise the total tissue content of AMP rises only slightly above the resting value of 0.1 μmol/g wet wt, or about 0.2 mM intracellularly. The free concentration of AMP, calculated assuming equilibrium with ATP, creatine phosphate and creatine through the creatine kinase and myokinase reactions, is <1 μM during rest and rises to several μM during exercise [1,2]. Thus in exercising muscle the concentration of IMP can exceed that of free AMP by a factor of 100 or more.

Activation of phosphorylase *b* by IMP was first reported in 1938 [3]. A physiological role for IMP in regulating the activity of phosphorylase *b* was ruled out on the grounds that the activation of the enzyme by IMP was weak when compared to its activation by AMP. Moreover, conversion of phosphorylase *b* to phosphorylase *a* seems to obviate the need for activation of phosphorylase during exercise. However, a report by Piras and Staneloni [4] showed that in rat skeletal muscle the conversion of phosphorylase *b* to *a* proceeds for only about 10 s after the start of strong exercise; thereafter phosphorylase *a* is converted back to the *b* form, even though exercise is continuing. We have confirmed this observation, finding that phosphorylase is largely converted back to the *b* form within 20 s after the start of exercise. Accumulation of IMP has reached a virtual maximum at this time. The large excess of IMP over free AMP that accumulates during exercise suggests that although IMP is a weaker activator of phosphorylase *b* than AMP, it may nevertheless be the physiological activator of the enzyme during sustained exercise and during recovery from exercise.

Dedicated to Professor Sir Hans Krebs, FRS, on his eightieth birthday

2. Experimental

2.1. *Exercise of leg muscle and preparation of extracts*

Male rats of the Sprague–Dawley strain were obtained from Charles River Breeding Laboratories, Wilmington, MA. The animals received food and water ad libitum, and weighing 175–250 g at the time of use. They were anaesthetized by intraperitoneal injection of sodium pentobarbital (5 mg/100 g body wt) and the skin from the right hind leg was removed. The sciatic nerve was exposed and a Dastre's electrode (Palmer Co., London, England) was attached around the nerve in its gluteal course. The leg was fixed to a platform at the ankle with adhesive tape and allowed to recover for 20 min. Isometric contractions were then induced by square-wave electrical pulses from a Grass SD9 Stimulator (Grass Instruments, Quincy, MA, USA). Pulses were of 1 ms duration at a frequency of 25/s. The voltage was 20 V. In other animals the sciatic nerve was stimulated with pulses of 10 ms duration at a frequency of 5/s and the voltage was maintained at 1–5 V. Muscles were exercised for various periods. A portion of the stimulated musculature, mainly gastrocnemius and posteroinferior thigh muscle, was rapidly frozen in situ with aluminium clamps cooled in liquid nitrogen. Controls were treated in a similar manner but were not exercised. Samples of frozen muscle were powdered in a stainless steel percussion mortar cooled in liquid nitrogen and used for measurements of phosphorylase activity and metabolite contents.

For the determination of phosphorylase activity, 50–100 mg of the frozen pulverized muscle were weighed and homogenized in 15 vol. of a pre-cooled

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solution of 60% glycerol containing 20 mM NaF, 1 mM EDTA and 20 mM glycerophosphate (pH 6.7) at −35°C in a bath of dry ice–50% ethylene glycol. The homogenization was continued until the powder was thoroughly dispersed but lasted <8 s. The sample was diluted 5-fold by adding a mixture containing 20 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, and 20 mM glycerophosphate (pH 6.7). The sample was immersed in an ice-water bath and homogenized again for 2 min. The homogenate was centrifuged at 1400 × g_{max} and 2°C for 10 min. Phosphorylase *a* and *b* activities were assayed in the supernatant.

For measurements of metabolite contents, samples of frozen muscle were homogenized at −10°C in 3.5 vol. of ice-cold 8% (v/v) perchloric acid in 40% (v/v) ethanol. A portion of the homogenate was used for the determination of glycogen and the remainder was centrifuged at 25 000 × g_{max} for 10 min. The supernatant was neutralized to pH 7 with a solution containing 2 M KOH and 0.5 M triethanolamine, the mixture was allowed to stand on ice for 15 min, and precipitated $KClO_4$ was removed be centrifugation. Metabolite contents were determined spectrophotometrically using double-beam spectrophotometers (Perkin-Elmer models 356 and 557) by the following enzymatic methods: glycogen [5], glucose 1-phosphate and glucose 6-phosphate [6], fructose bisphosphate and glyceraldehyde 3-phosphate plus dihydroxyacetone phosphate [7], lactate [8], ATP and creatine phosphate [9], ADP [10], and IMP [11].

2.2. *Assay of phosphorylase* a *and* b *in muscle extracts*

Muscle extracts prepared as described above were assayed for phosphorylase activity in reaction mixtures containing 10 mM potassium phosphate (61% dipotassium), 0.125 mg glycogen/ml, 50 mM imidazole–HCl buffer (pH 7.0), 2.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 μM glucose 1,6-bisphosphate, 0.5 mg NADP/ml, 5 μg phosphoglucomutase/ml, 3.8 μg glucose 6-phosphate dehydrogenase/ml and different concentrations of AMP, in a total vol. of 2 ml. The reaction was started by addition of 10 μl muscle extract. The temperature was 30°C. The reaction was followed by measuring $\Delta A_{340} - \Delta A_{400}$ with a Perkin-Elmer model 356 or 557 two-wavelength spectrophotometer.

In earlier studies phosphorylase activity assayed in the absence of AMP was presumed to represent the amount of phosphorylase *a*, while activity assayed in the presence of high concentrations of AMP was presumed to represent the sum of the *a* and *b* forms. However, phosphorylase *a* can have a considerable dependence on AMP [12]. As shown in fig.1, under our assay conditions phosphorylase activity rises sharply upon adding several μM AMP. This rise is due to phosphorylase *a*. A more gradual, linear rise in activity occurs at >20 μM AMP; this is due to phosphorylase *b*. The activity of phosphorylase *a* is therefore best estimated by extrapolation of the gradual rise in activity to zero AMP. The dependence of phosphorylase activity on μM levels of AMP was not altered appreciably by raising the glycogen concentration 8-fold. Routine assays for phosphorylase *a* and *b* were run in the presence of 0, 2, 20, 40 and 500 μM AMP. Activities of phosphorylase *a* and *b* were calculated as follows:

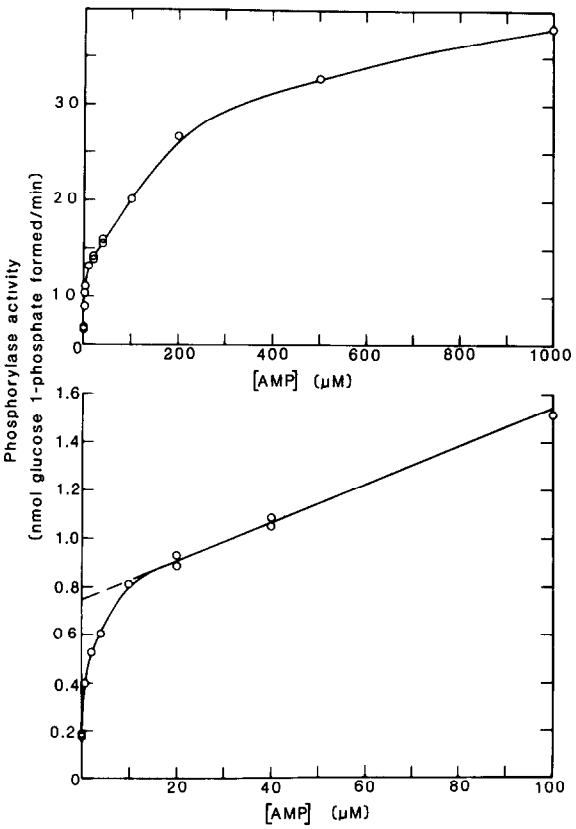

Fig.1. Effect of AMP on a mixture of phosphorylase *a* and *b* in an extract prepared from resting rat muscle. The reaction mixture for the assay of phosphorylase *a* and *b* was as given in section 2; AMP was varied as indicated.

Phosphorylase *a* (extrapolated) = $v_{ex} = 2v_{20} - v_{40}$

where v is the enzymatic rate at the μM levels of AMP shown in the subscript.

Phosphorylase $b = (v_{500} - v_{ex}) \times 1.77$

Values of v_o and v_2 were about 20% and 60% of v_{ex}, respectively. Previous reports showed that phosphorylase *a* has a K_{app} value for AMP in the μM range [12,13]. When the proportion of phosphorylase *a* was very low, the extrapolation method was not suitable, and the activity of the *a* form was estimated from v_2.

Earlier, using determinations of the percentage of phosphorylase *a* assays with and without AMP are not necessarily inaccurate. In most of these studies phosphorylase was assayed in the reverse direction with high concentrations of glucose 1-phosphate, conditions under which phosphorylase *a* may exhibit less dependence on AMP. Furthermore, the contribution of endogenous AMP may be significant. The total amount of AMP in muscle is about 0.1 μmol/g [1,2]. In our procedure, the muscle is diluted about 80-fold in making the extract and again by a factor of 200 into the assay reaction mixture, for a total dilution factor of 16 000; therefore, the endogenous contribution of AMP is about 0.01 μM. However, in some earlier studies the total dilution factor was only about 200, which might lead to about 0.5 μM AMP in the reaction mixture. Under some conditions the K_{app} for AMP for phosphorylase *a* is less than 0.5 μM [12].

The factor of 1.77 used for the ratio of V_{max} to v_{500} for phosphorylase *b* was estimated by curve fitting to data obtained with an extract containing very little phosphorylase *a* (fig.2). The curve fitting is complicated by the inhibition of phosphorylase *b* by mM levels of AMP [14]. With the simple formulation of the inhibition given in the legend to fig.2, a better fit to the data in the inhibitory range can be obtained by using a value for V_{max} about 25% lower; however, the fit to the data at lower AMP concentrations is then poorer, even with adjustment of the other parameters.

2.3. *Assay of phosphorylase* b *in the presence of glucose 6-phosphate*

The reaction mixture contained 10 mM potassium phosphate (61% dipotassium salt), 1 mg glycogen/ml, 10 mM ATP, 0.5 mM glucose 6-phosphate, 50 mM imidazole–HCl buffer (pH 7.0), 114 mM KCl, 11 or 18 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mg bovine serum albumin/ml, 15 μg crystalline rabbit muscle phosphorylase *b*/ml, and AMP or IMP as indicated, in a total volume of 1 ml. The ATP contributed 20 mM Na^+, 20 mM K^+, and 8 μM IMP. The assay was at pH 6.8 and 38°C. The reaction was started by adding the

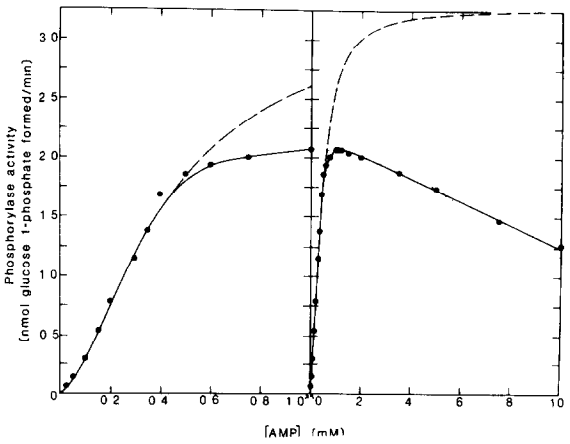

Fig.2. Effect of AMP on phosphorylase *b* activity in an extract from rat muscle. The assay conditions were as described in fig.1, except that the extract used was from a muscle which had been strongly exercised (25 pulses/s) for 1 min and which contained <1% phosphorylase *a*. The dashed line is a curve calculated from the equation:

$$v = V_{max} \times [AMP]^n / ([AMP]^n + S_{0.5}^n)$$

with V_{max} = 3.28 nmol/min, $S_{0.5}$ = 0.428 mM and n = 1.6. The dotted line shows the effect of including in the calculation a term for inhibition by AMP, such that the inhibited rate $v_i = v/(1 + [AMP]/K_i)$, with K_i = 5.9 mM.

enzyme. At appropriate intervals ranging from 10–60 min, 0.2 ml samples of the reaction mixture were added to 3.6 ml of a solution containing 55.5 mM Tris buffer (50% neutralized with HCl) and 1.11 mM $MgCl_2$ at 0°C. The diluted sample was frozen and stored until assayed as follows. To 1.9 ml of each sample were added 0.1 ml NADP (10 mg/ml) and 0.04 ml 25 μM glucose 1,6-bisphosphate. Glucose 6-phosphate and glucose 1-phosphate were determined by the sequential addition of glucose 6-phosphate dehydrogenase (2.5 μg in 10 μl) and phosphoglucomutase (2.5 μg in 5 μl). The reaction was run at 30°C and was followed by measuring $\Delta A_{340} - \Delta A_{400}$ with a Perkin–Elmer model 356 spectrophotometer. The rate of the phosphorylase reaction was then calculated from the amount of glucose 1-phosphate produced in the sampling time interval.

Tris has a pK of about 8 at 30°C. At pH 8 phosphoglucomutase and glucose 6-phosphate dehydrogenase are quite active but phosphorylase is strongly inhibited [15]. The pK of Tris is substantially higher at 0°C. Freezing and thawing at the high pH largely

inactivates phosphorylase, and there was little or no detectable increase in absorbance once the glucose 1-phosphate assay had been completed. Under these conditions the amount of glucose 1-phosphate assayed was proportional to the amount of phosphorylase used and to the sampling time, up to about 0.5 μmol/ml of the undiluted reaction mixture. Thereafter, the rate declined due to consumption of the glycogen. For this reason a higher glycogen concentration was used here than in the standard continuous assay for phosphorylase.

2.4. *Materials*

Glycogen (from rabbit liver, type III), glucose 6-phosphate, glucose 1,6-bisphosphate, dithiothreitol, D,L-α-glycerophosphate, and Tris base were obtained from Sigma. AMP, IMP, and NADP were from P-L Biochemicals. APP was obtained from Boehringer-Mannheim, imidazole from Eastman Organic Chemicals and bovine serum albumin from Pentex. Analytical enzymes were from Boehringer-Mannheim or Sigma.

Enzymatic analysis showed that the ATP and IMP contained 0.08 and 0.02 mol% AMP, respectively. Stock solutions were therefore treated with adenylate deaminase (prepared by Mr Michael Garabedian as described in [16]) to convert the AMP to IMP. The glycogen contained <0.1 nmol AMP/mg.

Phosphorylase *b* was prepared from rabbit muscle by Mr Mien-Chie Hung according to the procedure of Fischer and Krebs [17] and recrystallized twice. On the day of use, 0.2 ml of the crystalline suspension was centrifuged and the crystals were dissolved in 3 ml 50 mM imidazole–HCl buffer (pH 7.0). The solution was recentrifuged to remove traces of undissolved material. The solution contained 0.25 mg protein/ml, as assayed by the method of Lowry et al. [18].

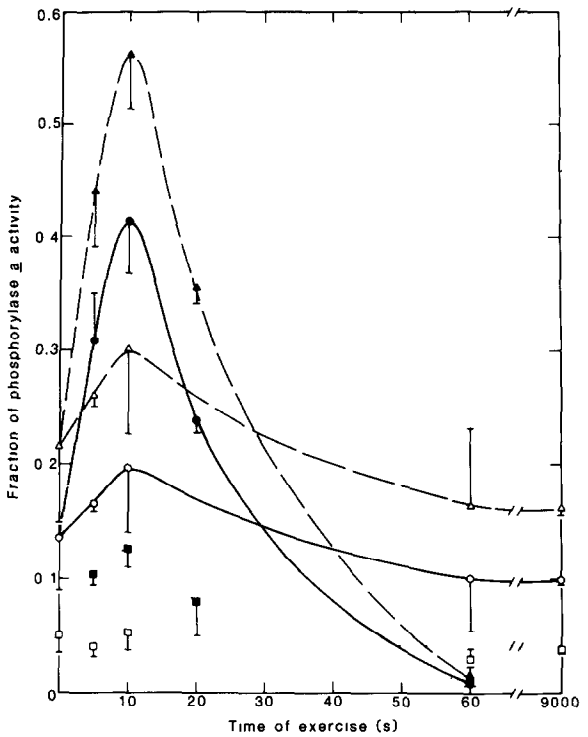

Fig.3. Effect of exercise on the fraction of phosphorylase in the *a* form in rat skeletal muscle. Muscles were stimulated at 5 pulses/s (open symbols) or 25 pulses/s (filled symbols) and assayed for phosphorylase activity in the presence of different concentrations of AMP. The fraction of phosphorylase *a* activity ($a/(a + b)$) is shown for three methods of calculation: (1) solid lines, phosphorylase *a* activity is extrapolated ($a = v_{ex} = 2v_{20} - v_{40}$) and $b = (v_{500} - v_{ex}) \times 1.77$, where v is the enzymatic rate at the μM level of AMP shown in the subscript; (2) dashed lines, phosphorylase *a* is extrapolated and $b = v_{500} - v_{ex}$; (3) dotted lines, phosphorylase *a* is the activity in the absence of AMP ($a = v_o$) and $b = v_{500} - v_o$.

3. Results

3.1. *Effect of exercise on the amount of phosphorylase* a

The fraction of phosphorylase in the *a* form in rat hind leg muscle at rest is about 14%. Exercise at 25 pulses/s results in a 3-fold increase in the fraction of phosphorylase *a* after 10 s (fig.3). However, by 20 s the fraction of phosphorylase *a* has dropped considerably, and by 60 s it is only a few percent and well below the resting value. If weaker conditions of exercise are used (5 pulses/s), there is only a slight increase in phosphorylase *a* above the resting value after 10 s exercise, and after 1 and 15 min the fraction of phosphorylase *a* is slightly below the resting value.

In the calculation of the above data, phosphorylase *a* activity was determined by extrapolating the enzymatic rates in the presence of 20 and 40 μM AMP to zero AMP. Furthermore, a factor of 1.77 was used to convert the activity of phosphorylase *b* measured in the presence of 500 μM AMP to V_{max}. If such a conversion factor is not used, the calculated fractions of phosphorylase *a* are somewhat higher at all time points (fig.3, dashed lines), but the shapes of the curves are unchanged. If the enzymatic rate in the

Table 1
Effect of exercise on content of selected metabolites of rat skeletal muscle

Metabolite (μmol/g dry wt)	Rest	Duration of exercise			
		5 s	10 s	20 s	60 s
Glycogen	78.6 ± 6.3	60.5 ± 1.9	51.2 ± 7.9	35.4 ± 11.7	9.1 ± 3.4
Glucose 1-phosphate	0.06 ± 0.01	0.54 ± 0.13	0.91 ± 0.21	1.22 ± 0.24	1.10 ± 0.01
Glucose 6-phosphate	0.83 ± 0.26	6.84 ± 1.64	11.1 ± 1.6	15.0 ± 2.2	18.38 ± 1.08
Fructose bisphosphate	0.03 ± 0.01	0.67 ± 0.17	1.83 ± 0.19	0.67 ± 0.22	0.07 ± 0.01
Triose phosphate	<0.001	0.45 ± 0.06	0.78 ± 0.17	0.30 ± 0.11	0.04 ± 0.01
Lactate	7.41 ± 1.19	20.5 ± 3.9	42.5 ± 9.7	76.0 ± 5.1	124.6 ± 6.1
Creatine phosphate	81.9 ± 8.6	36.6 ± 2.6	30.7 ± 5.8	15.4 ± 1.2	8.1 ± 0.2
ATP	23.9 ± 3.1	20.6 ± 2.8	21.3 ± 3.3	19.8 ± 1.8	14.6 ± 0.7
ADP	3.07 ± 0.10	3.18 ± 0.36	3.29 ± 0.26	3.18 ± 0.60	2.85 ± 0.48
IMP	0.052 ± 0.013	0.89 ± 0.39	3.01 ± 1.50	3.91 ± 1.47	4.42 ± 1.21

Isometric contractions were induced by square-wave electrical pulses of 1 ms duration at a frequency of 25/s. The voltage was 20 V. Other conditions are described in section 2. Metabolite contents are expressed as means ±SE. Four animals were analyzed for each group. 'Triose phosphate' means glyceraldehyde 3-phosphate plus dihydroxyacetone phosphate. The content of creatine phosphate plus creatine was 146 μmol/g dry wt.

absence of AMP is taken to represent the phosphorylase *a* activity, then the calculated fractions of phosphorylase *a* are decreased several fold at all time points (fig.3, dotted lines), because of the dependence of phosphorylase *a* activity on AMP (fig.1).

3.2. *Effect of exercise on glycogen, glycolytic intermediates, and nucleotide content*

The conversion of phosphorylase *b* to *a* during the initiation of exercise is associated with a rapid decrease in the glycogen content and a large rise in glucose 1-phosphate and glucose 6-phosphate (table 1). The mass action ratio of the phosphoglucomutase reaction is maintained between 12 and 17, close to the equilibrium value of 17 [19]. Although phosphorylase *a* starts to be converted back to *b* after 10 s, glycogenolysis continues and the glycogen content is reduced to 12% of the resting value after 60 s of exercise. Activation of glycolysis is indicated by the large increases in fructose bisphosphate, triose phosphate, and lactate by 5 s. Lactate continues to accumulate, but the levels of fructose bisphosphate and triose phosphate decrease after 10 s and are quite low at 60 s. After the first 10 s of exercise, the increase in glycolytic intermediates is 10–20% higher than the decrease in glycogen. This difference may reflect uptake and utilization of blood glucose.

The strong muscular exercise leads to a decrease in creatine phosphate of 50% after 5 s and of 90% after 60 s. There is a smaller percentage decrease

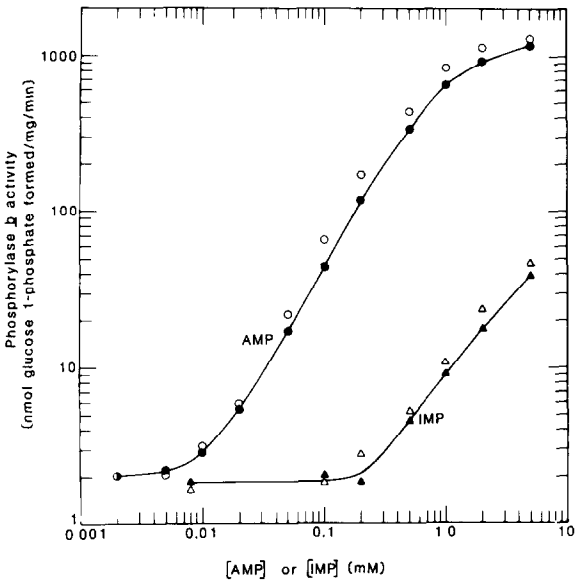

Fig.4. Activation of phosphorylase *b* by AMP and IMP in the presence of ATP and glucose 6-phosphate. The reaction mixture for the assay of phosphorylase *b* in the presence of glucose 6-phosphate was as given in section 2. The $MgCl_2$ concentration was either 11 mM (●,▲) or 18 mM (○,△).

in ATP, and ADP remains constant. Exercise causes a 17-fold increase in the content of IMP in 5 s. The IMP content is 3 μmol/g dry weight (in excess of 1 mM) after 10 s and it increases more slowly thereafter. Conditions of weaker exercise (5/s) which give much less change in phosphorylase *a* (fig.3) never-

theless cause large changes in the levels of IMP, lactate, and creatine phosphate [1,20].

3.3. *Activation of phosphorylase* b *by IMP*

Fig.4 shows the activation of rabbit muscle phosphorylase *b* by IMP and AMP in the presence of 10 mM ATP and 0.5 mM glucose 6-phosphate. ATP and glucose 6-phosphate inhibit phosphorylase *b* [14] and concentrations of these inhibitors that are typical of resting muscle were chosen for this study. A total concentration of 11 mM $MgCl_2$ (filled symbols) gives a free magnesium concentration of about 1 mM, which is close to the intracellular value (calculated from muscle contents of citrate and isocitrate [20]). Under these inhibitory conditions the K_{app} for AMP is about 1 mM and the activity of phosphorylase *b* in the absence of AMP is <0.2% of its activity at 5 mM AMP; this low level of glucose 1-phosphate production is close to the limit of detection of the method. The activity of the enzyme in the absence of added AMP is not due to contamination of the ATP or the glycogen by AMP (see section 2), nor is it due to contamination of the enzyme with phosphoglucomutase, since a similar basal activity was observed in the absence of added glucose 6-phosphate. A basal activity of 3% has been reported under much less inhibitory conditions [21]. The enzyme shows a slightly higher affinity for AMP in the presence of a total concentration of 18 mM $MgCl_2$ (open symbols) which gives a free magnesium concentration of a little less than 8 mM (since some magnesium is bound to phosphate as well as to ATP). Such an effect of magnesium was reported in [22]. It is necessary to use 50-times as much IMP in order to obtain the same degree of activation as is obtained with AMP (fig.4). Note that concentrations of 1–2 mM IMP are produced by exercising muscle (table 1) under conditions when the free AMP concentration is likely not to exceed 10–20 μM.

3.4. *Activation of phosphorylase* a *by IMP*

A muscle extract with a high proportion of phosphorylase in the *a* form was used in the experiment to be described. Fig.5 shows that in the presence of physiological concentrations of Mg^{2+} and K^+, IMP can cause a several-fold increase in the activity of phosphorylase *a* in muscle extracts. Again, it is necessary to use about 50-times as much IMP to obtain the same degree of activation as is obtained with AMP; at 1 mM IMP the activation is about 50% of that observed with 0.1 mM AMP.

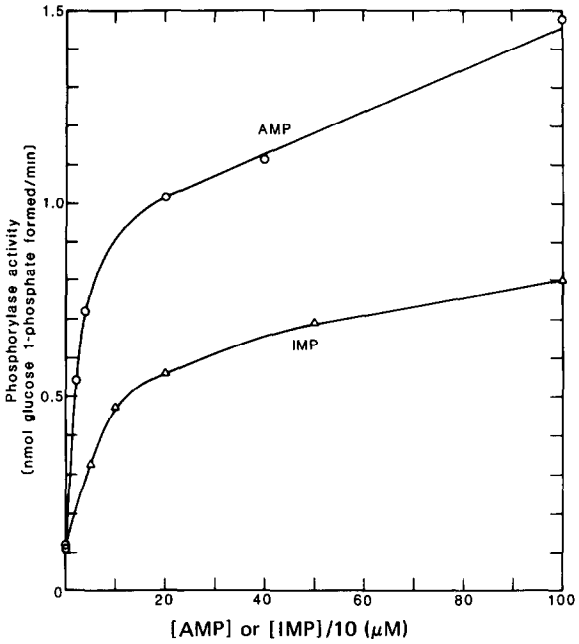

Fig.5. Effect of IMP and AMP on phosphorylase activity in an extract from exercised rat muscle. The assay conditions were as described in fig.1, except that the reaction mixture contained 1 mM $MgCl_2$, 134 mM KCl, and AMP or IMP as indicated. The reaction was started by addition of 10 μl of an extract of exercised muscle prepared as described in section 2; this extract contained about 40% phosphorylase *a*, as determined in the standard assay. The large increase in phosphorylase activity at low concentrations of AMP or IMP is due to activation of phosphorylase *a*; the more gradual increase in activity at higher concentrations of nucleotide is due to activation of phosphorylase *b*.

4. Discussion

The amount of phosphorylase in the *a* form in resting muscle is about 15% of the total activity. It rises about 3-fold after the onset of strong exercise. By 60 s the amount of phosphorylase *a* is considerably less than the resting value (fig.3). Similar observations were reported by Piras and Staneloni [4] and, while this manuscript was in preparation, by Conlee et al. [23]. With milder exercise there is little increase in phosphorylase *a* over the resting value (fig.3), but this too begins to decline again after 10 s. The following questions are raised by these observations:

(i) Why is the rate of glycogenolysis increased greatly when the amount of phosphorylase *a* rises only 3-fold?

(ii) Why is the phosphorylase *a* that is present in resting muscle apparently inactive?

Table 2
Calculated concentration of free AMP in muscle at rest and during exercise

Status of muscle	[ATP] (mM)	[Creatine] / [Creatine-P]	$[AMP]_{free}$ (μM) pH 7.0	pH 6.6	pH 6.2
Rest	9.6	0.78	0.22	0.043	0.010
Exercise 5 s	8.2	2.94	2.7	0.54	0.12
Exercise 10 s	8.5	3.7	4.4	0.85	0.20
Exercise 20 s	7.9	8.5	21	4.2	1.0
Exercise 60 s	5.8	17.2	67	13	3.1

Free AMP concentrations were calculated from the data of table 1 using the equation:

$$[AMP]_{free} = [ATP]([creatine]/[creatine\ phosphate])^2 \times MF$$

The multiplication factor, *MF*, is 3.8×10^{-5} at pH 7.0, 7.4×10^{-6} at pH 6.6, and 1.75×10^{-6} at pH 6.2. The format of this equation [24] is more appropriate for calculations from tissue metabolite data than the equations given by McGilvery and Murray [25], which were designed for computer simulations. Concentrations in mM are obtained by multiplying tissue contents in μmol/g dry wt by 0.4; this assumes that the intracellular water is 50% of the wet weight and that the wet weight/dry weight ratio is 5. The content of creatine phosphate plus creatine is 146 μmol/g dry wt. The *MF* incorporates the equilibrium constants of the creatine kinase and myokinase reactions, the magnesium, potassium and proton binding constants of ATP, AMP and creatine phosphate, and the assumed K^+ and free Mg^{2+} concentrations of 150 mM and 1 mM, respectively [24]. (The equation in the footnote on p. 521 of [24] for the apparent equilibrium constant of the creatine kinase reaction should read: $K_{ck} = [H^+] \cdot K_{10} \cdot t/(d \cdot c)$.) The values of the constants were the same as those used in [1], except that the value used for K_9 was 0.240 (recalculated from earlier data [26]). Potassium binding constants were taken from Smith and Alberty [27]; constants not given by these authors were assumed to be similar to the sodium binding constants quoted by Kuby and Noltmann [28]

(iii) Why does glycogenolysis continue, albeit at a lower rate, when the phosphorylase *a* level drops below the resting level?

The activities of phosphorylase *a* and *b* are affected by a variety of activators and inhibitors and their possible involvement in the control of the activities of these two forms of the enzyme will now be examined briefly.

AMP is an activator of both the *b* and the *a* form of the enzyme. However, as has already been mentioned, the total AMP content of the hind leg muscle of rat changes little between rest and exercise. Table 2 shows free AMP concentrations calculated from the ATP, creatine phosphate, and creatine contents given in table 1, assuming that the creatine kinase and myokinase reactions are in equilibrium. The calculated AMP concentration depends on the pH. For resting muscle the intracellular pH is assumed to be 7.0. Since strong exercise lowers the intracellular pH, free AMP concentrations were also calculated for pH 6.6 and 6.2. The calculated free AMP concentration is <1 μM in resting muscle; it rises to several μM during exercise. Such low concentrations of AMP will, by themselves, have little effect on the activity of phosphorylase *b* (fig.4).

IMP accumulates rapidly in exercising muscle, reaching a concentration of 1.2 mM in 10 s (3.01 μmol/g dry wt/(2.5)) (table 1). Phosphorylase *b* is activated several fold by 1.2 mM IMP (fig.4). The effect of IMP on phosphorylase *b* was examined in several previous studies [21,29–32], but these were usually conducted under conditions of limited value for assessing whether IMP is a physiological activator of phosphorylase *b*. The experiment shown in fig.4 was performed in the direction of glycogen breakdown and in the presence of physiological concentrations of orthophosphate, ATP, glucose 6-phosphate, Mg^{2+} and K^+. However, under the severely inhibitory conditions shown in fig.4, 1 mM IMP activates phosphorylase *b* activity by only about 1% of V_{max}, which is not sufficient to account for the rates of glycogenolysis observed during exercise.

The average rates of glycogenolysis in μmol hexose . g fresh wt^{-1} . min^{-1} obtained from the glyco-

gen levels in table 1 are 43 for the first 5 s, 22 for the next 5 s, 19 for the next 10 s, and 8 for the next 40 s. The total phosphorylase activity (*a* plus *b*) measured under the standard assay conditions was 30 μmol . g fresh wt^{-1} . min^{-1}; an activity of 45 μmol . g fresh wt^{-1} . min^{-1} was obtained when the glycogen concentration of the assay was increased 8-fold. These assays were performed at 30°C, whereas the body temperature of the rat is 38°C.

ATP and glucose 6-phosphate both inhibit phosphorylase *b* [14]. The former falls by 11% and the latter rises 13-fold during the first 10 s exercise. Some other factors not considered here probably increase the affinity of the enzyme for activators. For example, basic substances such as protamine greatly increase the activation of phosphorylase *b* by IMP [33]. In any case, although the affinity of phosphorylase *b* for IMP is 2% of its affinity for AMP (fig.4), the concentrations of IMP produced in exercising muscle are 100–1000-times greater than the free AMP concentration, and therefore IMP should be the more effective activator.

The *a* form of phosphorylase also shows a considerable, though not absolute, dependence on AMP or IMP (fig.5), which may account for the apparent inactivity of the sizable amount of phosphorylase *a* in resting muscle. The dependence on AMP is very pronounced at low substrate concentrations, especially at 38°C [12]. Activation of phosphorylase *a* by IMP was shown in the direction of glycogen synthesis [34]. The concentrations of free AMP (table 2) or IMP (table 1) produced in 5 s of exercise would be sufficient to activate phosphorylase *a* 5-fold under the conditions used for fig.5. However, the affinity of the enzyme for AMP is decreased by ATP and by raising the temperature to 38°C (not shown in fig.5). Glucose 6-phosphate may have a similar effect. Although IMP is a weaker activator than AMP, its much higher concentration during exercise makes it likely that IMP is responsible for most of the activation. Regardless of whether IMP or AMP is considered to be the activator, phosphorylase *a* and *b* should no longer be regarded as the activator-independent and dependent forms of the enzyme, but rather as high affinity and low affinity forms. This fits the observation that changes in the rate of glycogenolysis caused by onset of exercise are much larger than the changes in the amount of phosphorylase *a*.

About 50% of the glucose equivalents lost from glycogen during the first 10 s strong exercise accumulate as hexose monophosphates (table 1). Fructose 6-phosphate was not measured; if the phosphoglucose-isomerase reaction is in equilibrium, fructose 6-phosphate levels can be expected to be ~30% of the glucose 6-phosphate level. Glucose 6-phosphate activates phosphorylase *a* phosphatase [35,36] and inhibits phosphorylase *b* kinase [32,37]. Thus the large increase in glucose 6-phosphate may be a major factor responsible for the decrease in phosphorylase *a* after 10 s strong exercise (fig.3). AMP inhibits conversion of phosphorylase *a* to *b*. IMP can also serve this function, but concentrations 100-times higher than for AMP are needed [38]. These effects are brought about by binding of glucose 6-phosphate, AMP, or IMP to phosphorylase and not to the kinase or the phosphatase. Other factors which may contribute to a lowering of phosphorylase *b* kinase activity are the decreases in glycogen and pH [32,39].

The conversion of phosphorylase *b* to *a* does not occur, or occurs at a reduced rate, in mice deficient in phosphorylase *b* kinase (I-strain mice). The high rate of glycogenolysis observed in these animals during exercise is believed to be largely due to the activation of phosphorylase *b* [40,41]. In muscle of I-strain mice the total AMP content changes little during exercise, but IMP accumulates rapidly and to higher levels than in normal mice [42,43]. These observations have been interpreted to show that IMP is the activator of phosphorylase *b* not only in muscle of I-strain mice, but also in normal muscle.

Calculation of the free AMP concentrations is based on the assumption that metabolites are evenly distributed in the tissue. Greater recruitment of some muscle fibres than of others would lead to larger local concentrations of free AMP than indicated by the calculations. However, such muscle fibres would also be expected to have higher concentrations of IMP, since the production of IMP is largely dependent on the availability of AMP as substrate for adenylate deaminase. Most of the AMP in muscle is not in equilibrium with the myokinase reaction and does not appear to be available to activate phosphorylase in muscle at rest; however, the possibility remains that part of this pool of AMP becomes available to activate the enzyme during exercise.

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